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GnRH receptor expression and reproductive function depend on JUN in the GnRH receptor-expressing cells

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Abbreviated title: Gonadotrope c-Jun regulates reproduction

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Abstract

Gonadotropin-releasing hormone (GnRH) from the hypothalamus regulates synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gonadotropes. LH and FSH are heterodimers, comprised of a common α-subunit and unique β-subunits, which provide biological specificity and are limiting components of the mature hormone synthesis. Gonadotrope cells respond to GnRH via specific expression of the GnRH receptor. GnRH induces the expression of gonadotropin genes and of the GnRH receptor by activation of specific transcription factors. The JUN (c-Jun) transcription factor binds to AP-1 sites in the promoters of target genes and mediates induction of the FSHβ gene and of the GnRH receptor in the gonadotrope-derived cell lines. To analyze the role of JUN in reproductive function *in vivo*, we generated a new mouse model that lacks JUN specifically in GnRH receptor-expressing cells (JUN-cKO). JUN-cKO mice displayed profound reproductive anomalies such as reduced LH levels resulting in lower gonadal steroid levels, longer estrous cycles in females, and diminished sperm numbers in males. Unexpectedly, FSH levels were unchanged in these animals, while GnRH receptor expression in the pituitary was reduced. Steroidogenic enzyme expression was reduced in the gonads of JUN-cKO mice, likely as a consequence of reduced LH levels. GnRH receptor driven Cre activity was detected in the hypothalamus, but not in GnRH neuron. Female, but not male, JUN-cKO mice exhibited reduced GnRH expression. Taken together, our results demonstrate that GnRH receptor expression levels depend on JUN and are critical for reproductive function.

Precis

Knock-down of JUN in the GnRH receptor-expressing cells leads to diminished reproductive capacity, reduced GnRH receptor expression and lower serum LH in male and female mice.

Introduction

Mammalian reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is the final brain output that regulates both expression and secretion of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gonadotropes (1). This function is mediated by gonadotrope-specific expression of the GnRH receptor, which belongs to the rhodopsin family of seven transmembrane G-protein coupled receptors (2). LH and FSH in turn stimulate steroidogenesis and gametogenesis in the gonads (3, 4).

Gonadotropin levels are primarily regulated by transcription of their unique β-subunits, which provide biological specificity. Alternations in the transcription of β-subunits correlate with changes in the concentration of the mature hormones in the circulation (5, 6). The β-subunits heterodimerize with a common α-subunit to form the mature glycoproteins (7). GnRH induces LHβ (*Lhb*), FSHβ (*Fshb*) and GnRH receptor (*Gnrhr*) transcription via induction of specific immediate-early genes: EGR1 that regulates *Lhb* transcription; and FOS and JUN, which activate both *Fshb* and *Gnrhr* transcription (4). The FOS and JUN transcription factors form the AP-1 heterodimer, which is rapidly and transiently activated (8). Both mouse and human *Fshb* and *Gnrhr* genes are induced by GnRH via AP-1 (9-13). Transcriptome analysis demonstrated that

AP-1 members are strongly induced by GnRH in LβT2 cells (14) and in primary rat gonadotrope cells (15).

Responsiveness of the *Fshb* gene to GnRH is conveyed by AP-1 response elements in the proximal promoter (9,16-19). GnRH induces FOS (c-Fos), FOSB, JUN (c-Jun) and JUNB, but not JUND in the LβT2 cell line, a model of mature gonadotropes. A combination of these factors binds the AP-1 site in the *Fshb* promoter (9). In the αT3 gonadotrope cell line, GnRH regulates *Gnrhr* expression via AP-1, as well (11, 20). JUN homodimer, or a heterodimer with FOS, FOSB, FRA1 or FRA2, binds the mouse *Gnrhr* promoter at two different sites (13, 21). AP-1 heterodimer of JUN and FOS also regulates expression of the human *GNRHR* gene by GnRH (22).

Although gonadotrope cell models, such as LβT2 and αT3 cells, facilitated identification of transcription factors that lead to induction of gonadotrope genes, it is critical to determine the roles of these transcription factors *in vivo*. LHβ induction by GnRH is mediated by the EGR1 transcription factor. EGR1 is an immediate early gene and a member of the zinc finger family of transcription factors. EGR1 plays a non-redundant role in reproduction, and other family members are unable to compensate. Consistent with this, global EGR1 knockout mice are infertile and lack LH expression resulting in blunted sex steroid hormone synthesis (23, 24). FOS also plays non-redundant roles in reproduction *in vivo* (25). In the pituitary, FOS is critical for gonadotropin gene expression, while expression of another glycohormone subunit, TSHβ (*Tshb*) is not affected. In the hypothalamus, FOS is expressed in both kisspeptin and GnRH neurons during the preovulatory surge and can be used as a marker of their activation (26-28). FOS is necessary for normal kisspeptin neuron numbers and *Kiss1* expression, primarily in the female, while GnRH neuron location, axon targeting or gene expression do not depend on FOS (25).

Since JUN is an obligatory heterodimerization partner of FOS for DNA binding (8), we used c-Jun$^{flox/flox}$ mice crossed to GnRH receptor Cre animals to create mice that lack JUN specifically in the GnRH receptor-expressing cells. These conditional knockout mice, JUN-cKO, were used to analyze the reproductive physiology and determine the cell-specific role of JUN in reproduction.

Materials and Methods

Cell lines and transient transfection

LβT2, a gift from Dr. Pamela Mellon (UCSD), were maintained in DMEM with 10% FBS at 37˚C and 5% CO_2. The line was authenticated with RT-PCR based expression analysis of endogenous gonadotropin β subunits. For transfection, LβT2 cells were plated in 12-well plates one day prior to transfection with FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN), 0.1 μg expression vectors, 0.5 μg of a luciferase-reporter plasmid (reported previously (17, 29-31)) and 0.1 μg of TK β-galactosidase, a reporter plasmid driven by a Herpes virus thymidine kinase (TK) promoter as a control for transfection efficiency. cDNA for the AP-1 transcription factors were in the same backbone under the same promoter, and their expression was evaluated by western blot. Forty-eight hours after transfection, cells were lysed with 100 nM KPO_4 buffer containing 0.2% Triton X-100 luciferase activity measured on a luminometer (Veritas Microplate luminometer from Turner Biosystems) by injecting 100 μl of buffer containing 25 mM Tris pH 7.8, 15 mM $MgSO_4$, 10 mM ATP, and 65 μM luciferin into each well. Using the Tropix Galacto-light β-galactosidase assay (Applied Biosystems, Foster City, CA) and following the manufacturer's instructions, β-galactosidase activity was measured subsequently. Transfections

were performed in triplicate and repeated a minimum of three times. 1-way ANOVA statistical analysis with Tukey's posthoc test was performed using the JMP program with significance set at $p<0.05$.

Animals

Mice lacking c-Jun in GnRH receptor-expressing cells were obtained by crossing c-Jun$^{flox/flox}$ mice with GnRH-Receptor-Cre (GRIC) mice. Briefly, c-Jun$^{flox/flox}$ mice, in which the only coding exon of the *c-Jun* allele is flanked by *LoxP* sites (32, 33), were created by Dr. Randall Johnson (UCSD, California). Gnrhr$^{tm1(cre)Uboe}$ mice (GnRH receptor-internal ribosome entry site-Cre, GRIC) carry a knock-in GnRH receptor allele fused to an internal ribosome entry site and a Cre transgene. GRIC drives Cre expression in pituitary gonadotrope cells (34). Since some Cre expression is also observed in male germ cells in these animals (35), the GRIC allele was always introduced via the female. Homozygous c-Jun$^{flox/flox}$ Cre+ mice served as experimental mice, while Cre- littermates were used as controls. TdTomato reporter mice, *Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J, were obtained from Jackson laboratory (strain 007909) and crossed to GRIC mice to analyze Cre activity. Animals were maintained under a 12-hour light, 12-hour dark cycle and received food and water *ad libitum*. All experiments were performed with approval from the University of California Animal Care and Use Committee and in accordance with the National Institutes of Health Animal Care and Use Guidelines using 8-week-old animals, unless indicated otherwise. Males and females were analyzed separately to determine potential sex differences. At least 6 animals per sex per genotype were analyzed and statistical differences between Cre+ and Cre- were determined by Student’s T-test and Tukey’s test for multiple comparison.

Fertility studies – 8-week-old Cre+ and Cre- male or female mice were individually paired with an adult C57BL/6 mouse of the opposite sex, and the presence of litters was monitored daily over a period of 4 months. Additionally, starting at 8 weeks of age, a separate cohort of female mice was assessed for estrous cycle stage with daily vaginal smears for 5 weeks.

Sperm count - The epididymides were dissected, macerated, incubated in 1 ml DMEM at room temperature for 30 minutes with shaking. Sperm was cleared with a 70 μm cell strainer, diluted with sterile water and counted with a haemocytometer.

Histological analyses and immunohistochemistry

Ovaries and testes were fixed overnight at 4°C in 4% paraformaldehyde or Bouin's fixative, respectively. Tissues were dehydrated in ethanol, embedded in paraffin, cut into 10 μm thick sections, floated onto UltraClear™ Plus Microslides (Denville Scientific Inc, Holliston, Massachusetts) and stained with hematoxylin and eosin.

Pituitaries were fixed in 4% paraformaldehyde, embedded in paraffin, and cut to 10 μm. Slides were deparaffinized in xylene and rehydrated. Antigen unmasking was performed by heating for 10 minutes in a Tris-EDTA-0.3% Triton X and endogenous peroxidase was quenched by incubating for 10 minutes in 0.3% hydrogen peroxide. Slides were then blocked with 20% goat serum and incubated with primary antiserum against LH (1:300 raised in rabbit, National Hormone and Peptide Program, NIDDK) overnight at 4°C. After PBS washes, slides were incubated with biotinylated goat anti-rabbit IgG (1:300, BA-1000, Vector Laboratories, Burlingame, CA) for 30 minutes. The Vectastain ABC elite kit (Vector Laboratories) was used per manufacturer's instructions, after which the DAB peroxidase kit was used for colorimetric staining. Slides were dehydrated in ethanol and xylene, and cover-slipped with Vectamount (Vector Laboratories).

To visualize costaining of TdTomato and pituitary hormones, pituitaries were fixed in 4% paraformaldehyde, frozen in OCT, and cut to 12 μm sections using Leica cryostat. Hypothalami were sectioned to 30 μm sections for GnRH staining. Slides were blocked with 20% goat serum and incubated with primary antibodies against LH or FSH (1:300 raised in rabbit, National Hormone and Peptide Program, NIDDK) or GnRH (provided kindly by Greg Anderson, University of Otago; Dunedin, New Zealand (36)) overnight at 4°C. After PBS washes, slides were incubated with biotinylated goat anti-rabbit IgG (1:300, BA-1000, Vector Laboratories, Burlingame, CA) for 30 minutes; followed by streptavidin-Cy5 (1:500, Molecular Probes, Thermo Fisher) for 30 minutes. Secondary antibody-only controls were performed and determined that endogenous TdTomato expression was strong for visualization and that its emission in the TdTomato/Rhodamine channel overlaps with FITC/Alexa 488 channel. Thus, Streptavidin-Cy5 was used for visualization of LH-, FSH- or GnRH-expressing cells and slides cover-slipped using Vectasheild (Vector Laboratories). To determine percent co-expression, we counted how many of the hundred LH- or FSH-containing cells express TdTomato and vice versa. We counted at least 3 non-overlapping fields of view in 3 different sections per mouse (=9 fields), and stained pituitaries from 3 male and 3 female Cre+ mice.

qPCR analyses

Tissues were dissected, total RNA extracted and reverse transcribed using Superscript III (Invitrogen, CA). qPCR was performed using an iQ SYBR Green supermix and an IQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA) with primers listed in Table 1 under the following conditions: 95°C for 15 min, followed by 40 cycles at 95°C for 20 sec, 56°C for 30 sec, and 72°C for 30 sec. A standard curve with dilutions of 10 pg/well, 1 pg/well, 100 fg/well, and 10

198 fg/well of a plasmid containing LHβ, or FSHβ cDNA, was generated in each run with the samples.
199 The amount of the gene of interest was calculated by comparing the threshold cycle obtained for
200 each sample with the standard curve generated in the same run. Replicates were averaged and
201 divided by the mean value of the GAPDH housekeeping gene in the same sample using $^{\Delta\Delta}$Ct
202 method. After each run, a melting curve analysis was performed to confirm that a single amplicon
203 was generated in each reaction. Statistical differences in expression between genotypes were
204 determined by Student's T-test, and Tukey's HSD for multiple comparisons using JMP software
205 (SAS Institute; Cary, North Carolina).
206
207 **Hormone analyses**
208 For serum collection, mice were sacrificed between 9-11 am by isoflurane inhalation and blood
209 was obtained from the inferior *vena cava*. The blood was left to coagulate for 15 minutes at room
210 temperature, and then centrifuged at 2000 RCF for 15 minutes for serum separation. Hormone
211 assays were performed by the University of Virginia, Ligand Core. The University of Virginia
212 Center for Research in Reproduction Ligand Assay and Analysis Core is a fee-for-service core
213 facility and is in part supported by the Eunice Kennedy Shriver NICHD/NIH Grant U54-HD28934.
214 LH was analyzed using a sensitive two-site sandwich immunoassay (37), and mouse LH reference
215 prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide program)
216 was used as standard. FSH was assayed by RIA using reagents provided by Dr. A.F. Parlow and
217 the National Hormone and Peptide Program, as previously described (38). Mouse FSH reference
218 prep AFP5308D was used for assay standards. Steroid hormone levels were analyzed using
219 validated commercially available assays, information for which can be found on the core's
220 website: http://www.medicine.virginia.edu/research/institutes-and-programs/crr/lab-

facilities/assay-methods-page and reported in (39). Limits of detection were 0.24 ng/ml for LH, 2.4 ng/ml for FSH, 3 pg/ml for estradiol, and 10 ng/dL for testosterone. Intra- and inter-assay coefficients of variation were 6.4%/8.0%, 6.9%/7.5%, 6.0%/11.4% and 4.4%/6.4% for the LH, FSH, estrogen (E2) and testosterone (T), respectively. For the assays used for this manuscript, inter-assay coefficients of variation data are the result of 30 assays for LH and FSH, and 60 assays for E2 and T. Six animals per group were used for each hormone analysis. Statistical differences in hormone levels between wild-type and null group were determined by Student's T-test, and Tukey-Kramer HSD for multiple comparisons using JMP software (SAS Institute; Cary, North Carolina).

Results

JUN induces FSHβ and GnRH receptor reporters in LβT2 gonadotrope cell line.

Given that the AP1 family of transcription factors is comprised of four FOS members (FOS (c-Fos), FOSB, FRA1 and FRA2) and three JUN members (JUN (c-Jun), JUNB and JUND), combinatorial heterodomerization of these provides a variety of different factors that can induce target genes. GnRH induces all family members in gonadotropes, except for JUND (9). Since AP-1 heterodimers bind FSHβ (*Fshb*) and GnRH receptor (*Gnrhr*) promoters using EMSA (9, 13, 21), we first analyzed the level of induction of these target genes in gonadotropes with different combinations of AP-1 factors. cDNAs for the AP-1 transcription factors were cloned in the same vector backbone under the same promoter, and their expression was confirmed by western blot (data not shown). We also compared the induction with AP-1 overexpression to the induction by GnRH (G, Fig. 1). Since GnRH receptor reporter induction by GnRH was previously analyzed

using αT3-1 cells, a model of immature gonadotrope, we determine the level of induction in the model of mature gonadotrope, LβT2 cells. GnRH induced FSHβ reporter 6.2 fold, and GnRH receptor reporter 2.4 fold. FRA1 (F1) and FRA2 (F2) did not induce FSHβ (Fig. 1A) or GnRH receptor (Fig. 1B) expression either alone, nor more highly in combination with either JUN or JUNB compared to JUN or JUNB alone. JUN in combination with FOS or FOSB induced FSHβ reporter to similar levels compared to the induction observed with GnRH treatment (Fig. 1A). JUN heterodimers induced FSHβ to higher levels compared to JUNB heterodimers with FOS or FOSB. GnRH receptor, on the other hand, was induced to similar levels by either JUN or JUNB heterodimers with FOS or FOSB (Fig. 1B). In LβT2 cells, GnRH receptor is induced by GnRH 2.4 fold, a similar level observed with AP-1 overexpression. Since JUN induces both AP-1 gene targets in gonadotrope-derived cell line, we next crossed c-JUN$^{flox/flox}$ mice to GnRH receptor Cre (GRIC) animals to analyze the role of JUN in gonadotropes *in vivo*.

Reduced reproductive capacity but normal gonadotrope numbers in mice lacking JUN in GnRH receptor-expressing cells. Previous studies successfully used the GRIC allele to express Cre recombinase in gonadotropes to analyze transcription factors' roles in gonadotropin gene expression (40-42). We used the GRIC allele to knockdown JUN and create a conditional JUN knockout (JUN-cKO). Because JUN is an immediate early gene that is expressed at a very low basal level, undetectable by immunostaining, we were unable to reliably demonstrate JUN knockdown in the gonadotrope. Thus, to analyze Cre activity in the gonadotrope, and co-expression of Cre and gonadotropin hormones, we used TdTomato reporter mice in which TdTomato is specifically induced in Cre-expressing cells, following Cre-mediated excision of the stop codon. Immunohistochemisty of frozen pituitary sections with antibodies to gonadotropin

hormones, revealed faithful expression of the TdTomato fluorescence; that 98% of TdTomato expressing cells also express LH or FSH, consistent with the previous report (34). Furthermore, 88% of cells that contain LH express TdTomato (Fig. 2A, white arrowheads indicate LH-containing cells lacking TdTomato expression). 76% of FSH-containing cells express TdTomato (Fig. 2B).

JUN-cKO animals exhibited profound changes in their reproductive physiology; females had significantly longer estrous cycles, 7.4 days, compared to 4.4 days per cycle in controls (Fig 3A, representative females' stage of the estrous cycle over a 33-day period; Fig 3B, days per cycle in 6 females per genotype; JUN-cKO, cKO; control, Ctr). Male JUN-cKO mice had a 43% lower sperm count compared to controls (Fig. 3C). JUN-cKO mice also displayed longer time intervals in between litters, when paired with wild type C57BL/6 mice of the opposite sex (Fig. 3D, female cKO data presented, male data not shown).

To assess the role of JUN in gonadotrope differentiation, we stained pituitaries from JUN-cKO and control mice for LH to determine the number of gonadotropes. The morphology and size of JUN-cKO (cKO, Cre+) and control (Ctr, Cre-) pituitaries were indistinguishable (Fig. 4A). We then counted gonadotropes and determined that animals of both sexes and both genotypes contained the same numbers of gonadotropes (Fig. 4B). Therefore, the lack of JUN in the gonadotropes did not affect gonadotrope numbers. The JUN-cKO and control animals were of the same size and weight (data not shown). Therefore, despite the same number of gonadotropes, the lack of JUN in gonadotrope cells results in subfertility in both sexes.

JUN-cKO mice have reduced LH levels. Analyses of gonadotropin levels in the circulation revealed that JUN-cKO males exhibited 49% lower serum LH compared to control males, while

290 the LH concentration in JUN-cKO diestrus females was reduced by 56% compared to control
291 females in diestrus (Fig 5A). Although GnRH induces FSHβ via FOS and JUN in the gonadotrope-
292 derived cell line, FSH levels were the same in both JUN-cKO and control males and in JUN-cKO
293 and control diestrus females (Fig 5B). Steroid hormone levels were reduced; testosterone was
294 lower in males, while estradiol was lower in females (Fig. 5C), likely due to reduced LH levels in
295 the circulation.

296 We also analyzed gonadotrope gene expression at 8 weeks of age. Consistent with the
297 reduction in LH concentration in the circulation, *Lhb* mRNA level was 29% lower in JUN-cKO
298 males (Fig. 6A) and 62% lower in JUN-cKO diestrus females compared to Cre- littermate controls
299 (Fig. 6B). Consistent with unaltered FSH levels, there was no difference in *Fshb* expression
300 between genotypes (Fig. 6C, D). Expression of the *Gnrhr* (GnRH receptor) mRNA, however, was
301 reduced by 28% in JUN-cKO males (Fig. 6E) and by 56% in JUN-cKO females (Fig. 6F).
302 Expression of the common *Cga* subunit (αGSU) that heterodimerizes with both LHβ and FSHβ
303 was unaffected (Fig. 6G, H). Previous studies analyzing *Lhb* expression did not reveal a role for
304 the FOS and JUN AP-1 family, while the importance of AP-1 in GnRH receptor induction is well
305 established (13,43). Our results may point to a role for AP-1 in *Lhb* expression. On the other hand,
306 concomitant reduction of both *Lhb* and *Gnrhr* expression in both JUN-cKO males and females
307 may implicate diminished GnRH receptor levels in lower *Lhb* mRNA. This is consistent with
308 previous studies postulating that the receptor concentration correlates with LHβ levels (44).
309

310 **Reduced LH target genes in the gonads of JUN-cKO mice.** We next analyzed potential
311 downstream effects of reduced LH levels in the gonads in both males and females at 12 weeks of
312 age. Male JUN-cKO mice had a 22% reduction in seminal vesicle weight compared to controls

(Fig. 7A), which is consistent with reduced intratesticular testosterone levels (Fig. 7B). We also examined the expression of steroidogenic enzymes, which are induced by LH signaling. While *Star* (Steroidogenic acute regulatory protein, StAR) expression was unchanged, expression of *Cyp11* and *Cyp17* was reduced by 20% and 25%, respectively (Fig 7C, D, E). Expression of the FSH target gene in the testis, *Shbg* (Sex hormone binding globulin; androgen-binding protein, ABP) was unaffected, consistent with the unperturbed FSH levels in the circulation (Fig. 7F). We observed lower sperm numbers, as shown above. Testosterone levels, that were reduced due to the reduction in LH concentration, are necessary for spermatogenesis and for the maintenance of the blood-testis barrier. Blood-testis barrier is established via expression of tight junction proteins from the Claudin family (45,46). Expression of claudin 11 (*Cldn11*) did not change (data not shown). Claudin 3 (*Cldn3*) expression is regulated by androgens (47), however despite a decrease in testosterone, expression of *Cldn3* was not significantly reduced (Fig. 7G, p=0.1). Given that sperm numbers in the epididymides were diminished, we assessed markers for several stages of spermatogenesis (48) and determined that the early stage spermatogenesis marker *Sycp3* to be unchanged, while later stage markers such as *Spert* and *Elp* were reduced in JUN-cKO males by 31% and 36% compared to the controls, respectively (Fig. 7H, I, J). Histological analyses of the testes uncovered small number of abnormal seminiferous tubules (~5%) lacking mature sperm in the JUN-cKO males (Fig. 7L). Thus, lack of JUN in GnRH receptor-expressing cells in JUN-cKO males causes lower expression of steroidogenic enzymes and reduced levels of the late stage spermatogenesis markers, corresponding to reduced sperm count.

The ovaries of JUN-cKO mice weighed 37% less than control ovaries and contained fewer corpora lutea (Fig. 8A, B, C). JUN-cKO females expressed 43% lower level of the LH target gene *Cyp17a1* (Fig. 8D), while the FSH target gene *Cyp19a1* (aromatase) was unchanged in the ovaries

(Fig. 8E). Given that antral stage of folliculogenesis is not affected corresponding to unaltered FSH, fewer corpora lutea may stem from reduction in prolactin levels, since prolactin is necessary for corpus luteum function in rodents (49, 50). We measured expression of prolactin (*Prl*) in the pituitary and determined that *Prl* mRNA is reduced by 67% in JUN-cKO female mice (Fig. 8F). Therefore, female as well as male gonads from JUN-cKO animals exhibit a phenotype corresponding to diminished reproductive capacity.

Cre activity in the hypothalamus. The lack of an effect on *Fshb* expression and FSH levels in the circulation was unexpected, given previous evidence in the literature. In addition to inadequate Cre activity in a portion of gonadotrope cells, other JUN family members such as JUNB, that is also induced by GnRH (9), may compensate for the loss of JUN. Although JUN and JUNB exert non-overlapping functions in other tissues as evidenced by the different phenotypes of the respective knockout mice (32, 51), they may be able to substitute for each other in this scenario. To assess a possible compensatory increase in JUNB expression, we analyzed the level of *Junb* mRNA in the pituitaries of JUN-cKO and of control males and females. In both sexes, JUN-cKO animals exhibited an increase in JUNB expression in the pituitary (Fig. 9). Therefore, JUNB increase may be able to compensate for the loss of JUN for *Fshb* but not for *Gnrhr* expression.

On the other hand, reduced expression of *Gnrhr* and *Lhb* may stem from extrapituitary sites. GnRH receptor is expressed in several hypothalamic nuclei and may be expressed in GnRH neurons themselves (52-58). We used TdTomato reporter mice to determine activity of Cre recombinase in the hypothalamus. We also performed immunostaining for GnRH to detect GnRH neurons and determine whether TdTomato is expressed in GnRH neurons following Cre excision of the stop codon. Coronal sections of the mediobasal hypothalamus demonstrated that TdTomato

was expressed in the arcuate nucleus in GRIC+ animals, while GnRH axon terminals were located in the median eminence (Fig 10A, GnRH, green; TdTomato, red). Staining of the preoptic area detected GnRH neurons in their expected location, while TdTomato-expressing cells were situated more laterally (Fig. 10B, GnRH, green; TdTomato, red). There was no overlap of the green and red fluorescence in any section from either male or female mice. We also performed qPCR on biopsy punched preoptic area and analyzed *Gnrh* expression. *Gnrh* expression did not differ in control and JUN-cKO male mice (Fig. 10C). However, *Gnrh* expression was reduced by 56% in the female JUN-cKO mice (Fig. 10D). Given that GnRH neurons of either sex did not express TdTomato reporter, we hypothesize that lower *Gnrh* mRNA levels in the female may stem from the upstream regulatory neurons that may be affected by either lack of JUN in GnRH receptor-expressing cells, or by lower estrogen levels.

Discussion

The molecular mechanisms of GnRH regulation of its target genes in pituitary gonadotropes have been previously examined primarily in cell lines and in primary cultures; however, a role of GnRH-induced transcription factors regulating gonadotrope genes *in vivo* is just beginning to emerge. As an immediate-early gene, JUN is rapidly induced in gonadotrope cells following GnRH treatment, both *in vivo* (59) and in model cell lines (14, 60). In these, JUN mediates GnRH induction of the FSHβ (*Fshb*) gonadotropin subunit (9) and of the GnRH receptor (*Gnrhr*) (12) by binding to the AP-1 site in the proximal promoters of these genes following dimerization with FOS. Herein, we examined the role of JUN in HPG axis gene expression *in vivo*, using c-Jun$^{flox/flox}$ mice, crossed to GRIC, in which Cre expression is driven by the GnRH receptor promoter. We demonstrate that

JUN expression in the GnRH receptor-expressing cells is necessary for normal reproductive function.

Mice lacking JUN in GnRH receptor-expressing cells exhibit a number of reproductive defects. Males have decreased *Lhb* and *Gnrhr* expression, which results in a decline in LH concentration in the circulation, and consequent reduction in testicular function, including lower expression of several steroidogenic enzymes, leading to reduced testosterone levels, smaller seminal vesicles and fewer mature spermatozoa. Females, as well, have lower LH, which results in longer estrous cycles, reduced expression of *Cyp17* steroidogenic enzyme and fewer corpora lutea in the ovaries. Reduced number of corpora lutea, despite normal numbers of antral follicles, may stem from diminished intra-ovarian steroid hormone levels due to lower expression of *Cyp17*. Alternatively, fewer corpora lutea may be a result of abrogated prolactin levels. Prolactin has a critical permissive role for LH action in the ovary, and is necessary for luteinization and corpus luteum function in rodents (49, 50). Reduced prolactin expression likely derives from decreased levels of steroid hormones. Estrogen strongly upregulates prolactin in females (61, 62). In males, expression of aromatase in the pituitary allows for testosterone conversion to estrogen, which then increases prolactin levels (63). Therefore, decreased estrogen may contribute to diminished prolactin expression and reduced number of corpora lutea. Previous studies analyzing regulation of *Lhb* expression failed to find a role for JUN, while JUN is involved in *Gnrhr* induction. Since *Lhb* expression is dependent on GnRH receptor numbers at the surface of gonadotropes (44), we believe that reduced levels of GnRH receptors are a cause of diminished LH levels. On the other hand, it is possible that AP-1 may play a role in *Lhb* expression *in vivo.*

Unexpectedly, FSH levels were unchanged in the cKO animals, although in LβT2 model cell line JUN mediates GnRH induction of the *Fshb* subunit (9). This may illustrate discrepancy

between cell models and *in vivo* function, as suggested before (64). GRIC model has been used in the recent literature to analyze a role of transcription factors in the gonadotrope (40-42). We determined a significant overlap between LH and TdTomato expression. Although difference in the percent coexpression of the reporters and LH between previously reported results (34) and results reported herein is small, it may stem from different levels of fluorescent reporter expression. 12% of LH-expressing cells lack TdTomato expression demonstrating insufficient Cre activity in these cells. The number of FSH-expressing cells which do not have sufficient Cre expression is higher, at 24%. It is possible that FSH-containing cells that do not express functional Cre are sufficient to maintain normal levels of FSH in the circulation. Especially since FSH can be constitutively secreted (65, 66) and thus, would be less dependent on the level of GnRH receptor expression. Lack of GnRH receptor expression in a portion of the FSH-containing cells was reported previously (35), although it was postulated that this population is present only during development. Data presented herein imply that FSH-containing cells without GnRH receptor persist in adulthood, which is consistent with several previous studies (67, 68). Compensation by JUNB may explain unchanged FSH levels as well, although in most tissues JUN and JUNB have opposing effects (69, 70). However, FSHβ was more highly induced by JUN heterodimers than JUNB heterodimers, while GnRH receptor induction was the same with either JUN or JUNB heterodimers with FOS or FOSB. The effect on GnRH receptor expression may indicate that either the GnRH receptor is more sensitive to the levels of JUN, or that JUNB cannot compensate for JUN to induce GnRH receptor expression.

Given that GRIC allele also drives Cre expression in the testes, in the germ cells (35), there is a concern that gonadal phenotype in the male mice may be caused by a lack of JUN in testes. However, that is unlikely for several reasons. Specific lack of JUN in male and female JUN-cKO

results in the same outcomes: lower expression of *Gnrhr* and *Lhb* mRNA in the pituitary, reduced LH in the circulation and diminished expression of LH-dependent genes in the gonads, resulting in lower sex steroid levels. In fact, in the female JUN-cKO, all these effects are exacerbated compared to the male JUN-cKO. Furthermore, known targets of AP-1 in the testes are not affected. Although AP-1 binding site was identified in the FSH receptor promoter, regulating expression of the FSH receptor by FSH (71), in the testes of JUN-cKO males FSH receptor expression is not affected (data not shown). AP-1 factors also play roles in tight junction formation and blood-testis barrier (45, 46). Blood-testis barrier, which is necessary for spermatogenesis and fertility, is established via expression of tight junction proteins, primarily Claudin 11 (72-74). Expression of Claudin 11 is unaltered in JUN-cKO mice (data not shown). Claudin 3, whose expression is regulated by androgens, forms the stage-specific basal barrier in mice (47). Despite a decrease in circulating testosterone, expression of Claudin 3 is not significantly changed either. Since late stage spermatogenesis markers are reduced, AP-1 may regulate spermatogenesis directly (75). Due to a lack of known AP-1 target genes in germ cells, we are not able to delineate if decrease in late stage spermatogenesis may be due to testicular expression of Cre or to reduced levels of LH and diminished testosterone. Therefore, since males and females JUN-cKO exhibit similar phenotypes, and Cre is not expressed in the ovary, the observed effects likely stem from the gonadotrope specific JUN knockdown.

Cre expression is driven by the GnRH receptor regulatory region, which is expressed in several other extrapituitary sites. GnRH receptors, in addition to pituitary gonadotrope, are expressed in the mediobasal hypothalamus, amygdala and hippocampus (52), but the specific neuronal populations that express GnRH receptors are not known. Several studies identified that GnRH receptor is expressed in about 50% of GnRH neurons (55-58), suggesting that GnRH

receptor may contribute to autocrine GnRH pulse generation (76, 77). Using this same GRIC mouse, ablation of GnRH receptor-expressing neurons resulted in elevated number of GnRH neurons (78), implying that GnRH receptor is not expressed in GnRH neurons themselves, but it may be expressed in afferent neurons that regulate GnRH neurons. It was also postulated that central GnRH via hypothalamic GnRH receptors upstream of GnRH neurons, may participate in the pulsatile release and preovulatory surge (79). Our analyses of GnRH receptor-driven Cre expression in the hypothalamus, demonstrated Cre activity in the arcuate nucleus and in the preoptic area, but not in GnRH neurons themselves. Examination of GnRH expression determined that *Gnrh* mRNA is significantly reduced specifically in female JUN-cKO. We previously observed female specific effects using FOS null animals (25). Since there was no overlap between GnRH neurons and TdTomato expression, these findings suggest that GnRH expression is mediated in part via activity-regulated gene induction by afferent neurons which may be affected by reduced estrogen levels or by JUN knockdown. A number of previous reports determined that hypothalamic factors involved in reproductive function, such as RFamide-related peptide 3 (RFRP-3), a mammalian gonadotropin-inhibitory hormone ortholog; senktide, a neurokinin B receptor agonist; and oxytocin; elicit changes in LH serum levels, not only via alterations of GnRH secretion but by modifications of *Gnrh* transcription (80-82). Alternatively, diminished *Gnrh* mRNA transcription may be secondary to reduced LH levels that caused lower estrogen (83, 84). Similar to other studies using whole animal models where endocrine loops are dysregulated, we are not able to distinguish between these alternatives. These results may indicate that the observed reproductive phenotype in females may stem from reduced GnRH expression.

In summary, our analyses of the mice that lack JUN in GnRH receptor-expressing cells revealed several physiological roles of this gene in the reproductive axis. Reduced GnRH receptor

and lower LH levels contribute to diminished sex-steroid hormone levels, impaired spermatogenesis and reduced numbers of corpora lutea. Unchanged FSH levels may be due to compensatory role of JUNB for this gene target but not for GnRH receptor, or to the presence of FSH gonadotropes that lack sufficient Cre activity. We demonstrate that JUN expression in GnRH receptor-expressing cells is necessary for normal reproductive function.

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Figure Legends

1. JUN-containing heterodimers induce both FSHβ and GnRH receptor reporters in LβT2 cells. Expression vectors for AP-1 isoforms were co-transfected with the mouse FSHβ (A) and GnRH receptor (B) reporters. In the separate sets of samples, cells transfected with the reporters were treated with vehicle (V) or GnRH (G, 10 nM GnRH, 5 hours). Data represents a mean of 3

independent experiments each performed in triplicate, and significant induction compared to the empty vector control is indicated with *.

2. TdTomato coexpresses with gonadotropin hormones. TdTomato reporter mice were crossed with GRIC to allow TdTomato expression in the Cre-dependent manner. Pituitaries from GRIC+/TdTomato- mice (not shown) and from GRIC+/TdTomato+ mice, from 4 separate litters, were sectioned and stained for LH and FSH. A, 88% of LH (green) cells coexpress TdTomato (red; while arrows indicate LH cells that do not express TdTomato); B, 76% of FSH cells coexpress TdTomato. One hundred gonadotropin hormone-containing cells were counted in 3 non-overlapping fields of view in 3 sections from 3 different male and 3 female mice.

3. Fertility is profoundly affected in JUN-cKO animals. A, Representative estrous cycle changes in Cre- control females (top) and JUN-cKO female (bottom) assessed by vaginal smears for 33 days starting at 8 weeks of age (E, estrus, P, proestrus, D/M (diestrus/metestrus). B, JUN-cKO animals (black bars) have increased average cycle length (6 females per group) than Cre-controls (gray bars). C, Sperm count indicates 43% lower numbers in 8-week old JUN-cKO compared to control littermates. D, Animals were continuously present in the cages with wild-type mice of opposite sex and monitored daily for litters. JUN-cKO mice had longer time interval between litters. * indicates difference between control (Ctr, gray bars) and JUN-cKO (cKO, black bars), determined by Student's T-test followed by Tukey's HSD test.

4. JUN is not required for gonadotrope differentiation. A, Pituitaries of control (Ctr, Cre-, $cJun^{flox/flox}$ homozygous without Cre recombinase) and JUN-cKO (cKO, Cre+, $cJun^{flox/flox}$

774 homozygous with Cre recombinase) were subjected to immunohistochemistry for LH to analyze
775 number of gonadotrope cells. B, quantification in males and females of gonadotropes indicates
776 that the lack of JUN has no effect on gonadotrope population (Ctr, Cre-, gray bars; cKO, Cre+,
777 black bars).
778
779 **5. Lower LH levels in JUN-cKO animals.** Six 8-week old control controls (Ctr, gray bars) and
780 six JUN-cKO littermates (cKO, black bars) were analyzed for serum gonadotropin concentration.
781 Females were monitored for the estrous cycle stage and analyzed in diestrus. Male and female
782 JUN-cKO mice have lower level of LH than Cre- controls (A), while FSH is unchanged (B).
783 Consequently, sex steroid levels are lower (C). Difference (*) between control (gray bars) and
784 JUN-cKO (black bars) were determined by Student's T-test followed by Tukey's HSD test.
785
786 **6. Reduced LHβ and GnRH receptor expression in JUN-cKO mice.** Pituitaries from six 8-
787 week old Cre- controls (Ctr, gray bars) and six JUN-cKO littermates (cKO, black bars) were
788 analyzed for expression of gonadotrope genes by qPCR: *Lhb* (LHβ; A-males, B-females), *Fshb*
789 (FSHβ; C-males, D-females), *Gnrhr* (GnRH receptor; E, F) and *Cga* (common αGSU; G, H).
790 Statistical significance (*) between control (gray bars) and JUN-cKO (black bars) were determined
791 by Student's T-test followed by Tukey's test.
792
793 **7. Reduced seminal vesicle weight and spermatogenesis in male JUN-cKO mice.** A, Seminal
794 vesicles were dissected and measured to reveal reduced weight in 12-week old JUN-cKO males.
795 B, Testes were homogenized and intratesticular testosterone measure. C-J, Testes were
796 homogenized and mRNA extracted using Trizol. qPCR revealed lower expression of CYP11 (D)

and CYP17 (E) steroidogenic enzymes and lower levels of mRNA for late stage spermatogenesis markers (I, J). K-L, Histological analyses of testes following H&E stain exhibits some abnormal seminiferous tubules in JUN-cKO males. Difference (*) between control (Ctr, gray bars) and JUN-cKO (cKO, black bars) were determined by Student's T-test followed by Tukey's HSD test.

8. Reduced expression of LH target gene CYP17 and fewer *corpora lutea* in JUN-cKO females. A, Histological analyses of ovaries following H&E stain illustrates lower number of corpora lutea in JUN-cKO females. B, Ovaries from JUN-cKO mice were smaller and C, had fewer corpora lutea. Ovaries were homogenized and mRNA extracted using Trizol. qPCR revealed lower expression of CYP17 (D) but not CYP19 (E) steroidogenic enzymes in 12-week old JUN-cKO female mice. F, Prolactin (*Prl*) expression in the pituitary was reduced. Statistical significance (*) between control (Ctr, gray bars) and JUN-cKO (cKO, black bars) were determined by Student's T-test followed by Tukey's posthoc test.

9. Increased JUNB expression. JUNB expression in the pituitaries of the 8-week old male and female control and JUN-cKO mice was analyzed to determine if JUNB expression is elevated in compensation for the lack of JUN. * indicates statistical significance determined by Student's t-test and Tukey's posthoc analysis.

10. Cre activity in the hypothalamus. A, Coronal sections at the level of the mediobasal hypothalamus demonstrate TdTomato reporter expression (red) and Cre activity in the arcuate nucleus, and GnRH axon terminals staining in the median eminence (green). B, Coronal section of the preoptic area shows that GnRH neurons (green) do not express TdTomato reporter (red).

Male and female mice from 4 separate litters were used, and no sex differences were detected. C, GnRH expression (*Gnrh*) in the hypothalami of male mice is not altered. D, Reduced expression of *Gnrh* gene in the female JUN-cKO mice. * indicates statistical significance determined by Student's t-test and Tukey's posthoc analysis.

Table 1. Antibodies

Antibody	Species	Dilution	Provider, cat # and RRID
LH	rabbit	1:300	NHPP, AFP240580Rb; RRID:AB_2665533
FSH	rabbit	1:300	NHPP, AFP-C0972881; RRID:AB_2687903
Prolactin	rabbit	1:300	NHPP, PRL; RRID:AB_2629220
GnRH	rabbit	1:5000	Greg Anderson, Univ. of Otago; RRID:AB_2721118

Table 2. **Primers**

Primers	Forward	Reverse
Lhb (LHβ)	CTGTCAACGCAACTCTGG	ACAGGAGGCAAAGCAGC
Fshb (FSHβ)	GCCGTTTCTGCATAAGC	CAATCTTACGGTCTCGTATACC
Cga (aGSU)	ATTCTGGTCATGCTGTCCATGT	CAGCCCATACACTGGTAGATGG
Gnrhr (GnRH receptor)	GCCCCTTGCTGTACAAAGC	CCGTCTGCTAGGTAGATCATCC
Prl (prolactin)	TGTTCCCAGCAGTCACCAT	CAGCAACAGGAGGAGTGTC
Star (StAR)	GGAGGGGTGGTAGTCAGGAGA	TCCCCTGTTCGTAGCTGCTG
Cyp11	AAGTATGGCCCCATTTACAGG	TGGGGTCCACGATGTAAACT
Cyp17a1	ATCCTTGTCACGGTGGGAGA	GGAGGTGAGTCCGGTCATTG
Cyp19a1 (aromatase)	TTCCCATGGCAGATTCTTGTG	CGAATCGGGAGATGTAGTG
Shbg (ABP)	GACATTCCCCAGCCTCATGCA	TGCCTCGGAAGACAGAACCAC
Cldn3 (claudin 3)	AACTGCGTACAAGACGAGACG	GGCACCAACGGGTTATAGAAAT
Gnrh (GnRH)	CTACTGCTGACTGTGTGTTTG	CATCTTCTTCTGCCTGGCTTC
Gapdh	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

Figure 1

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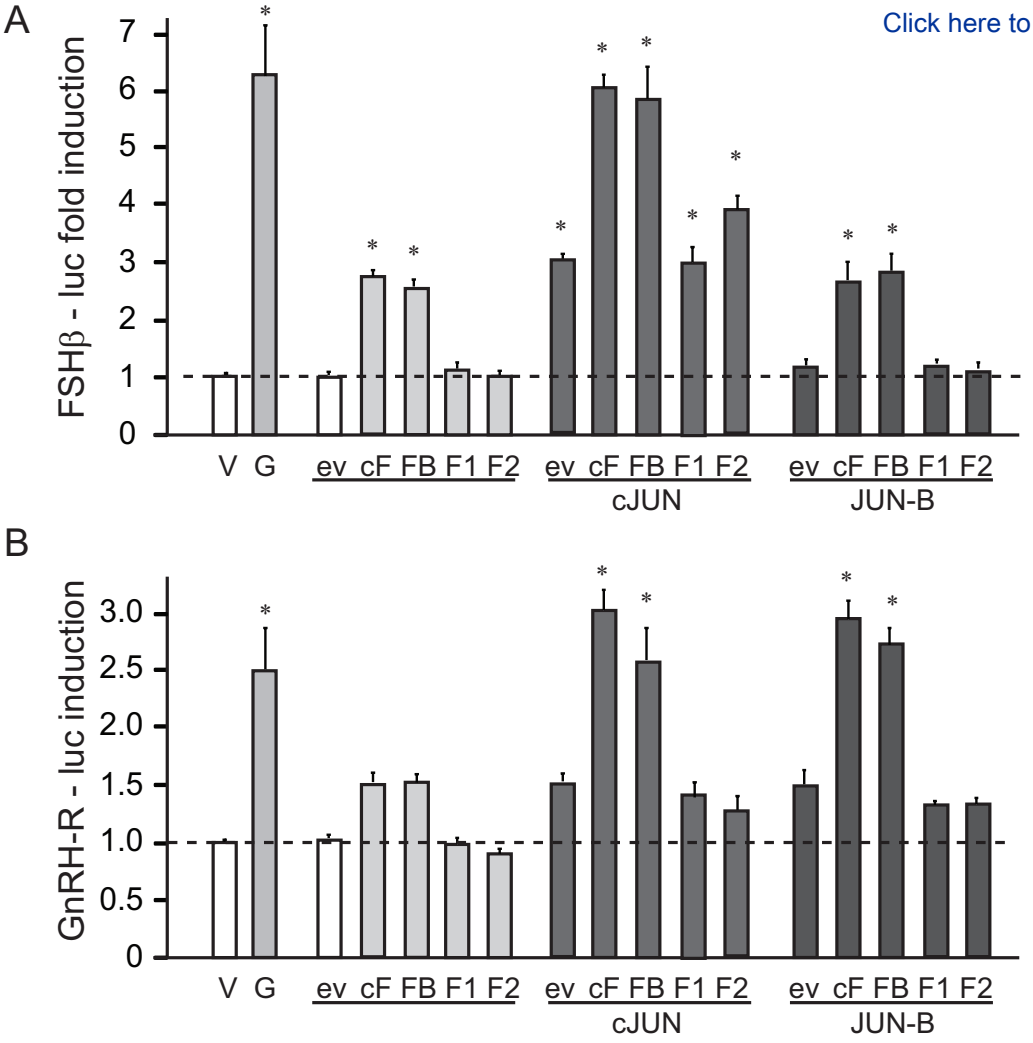

Figure 1

Figure 2

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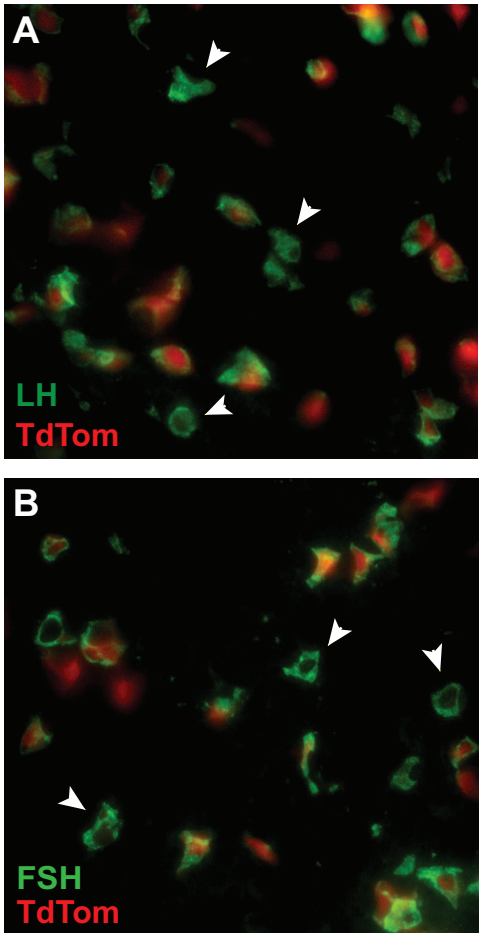

Figure 2

Figure 3

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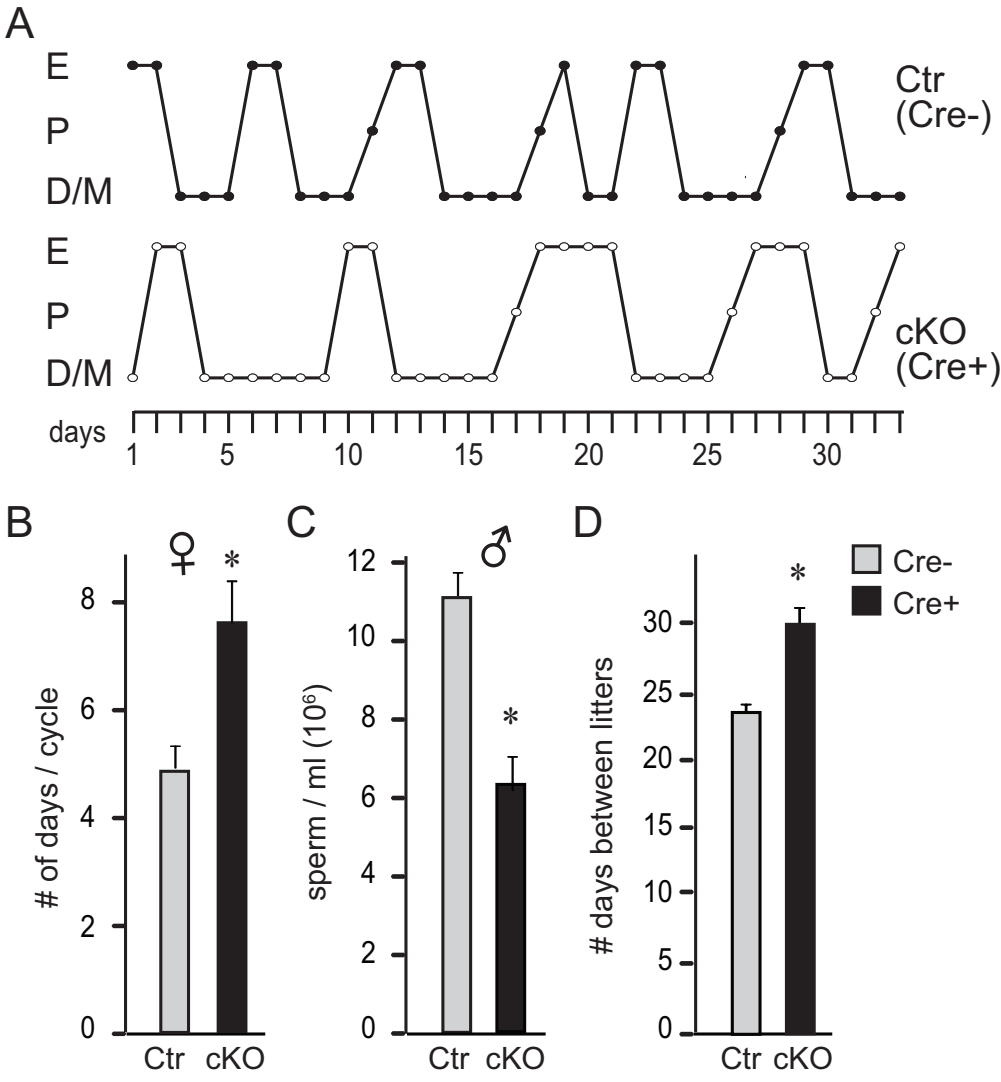

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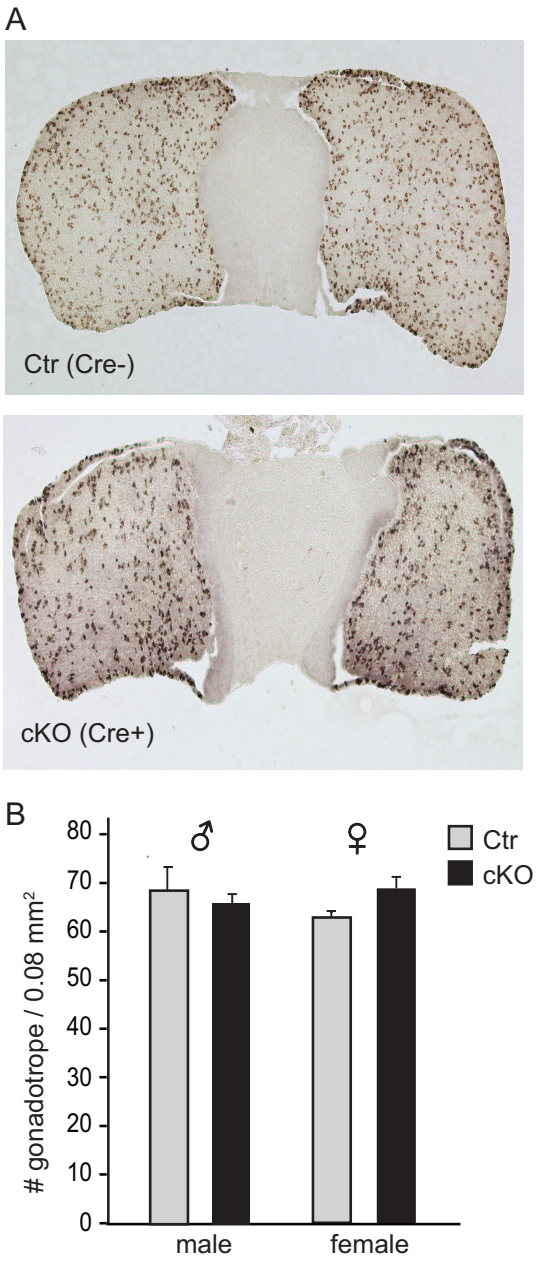

Figure 4

Figure 5

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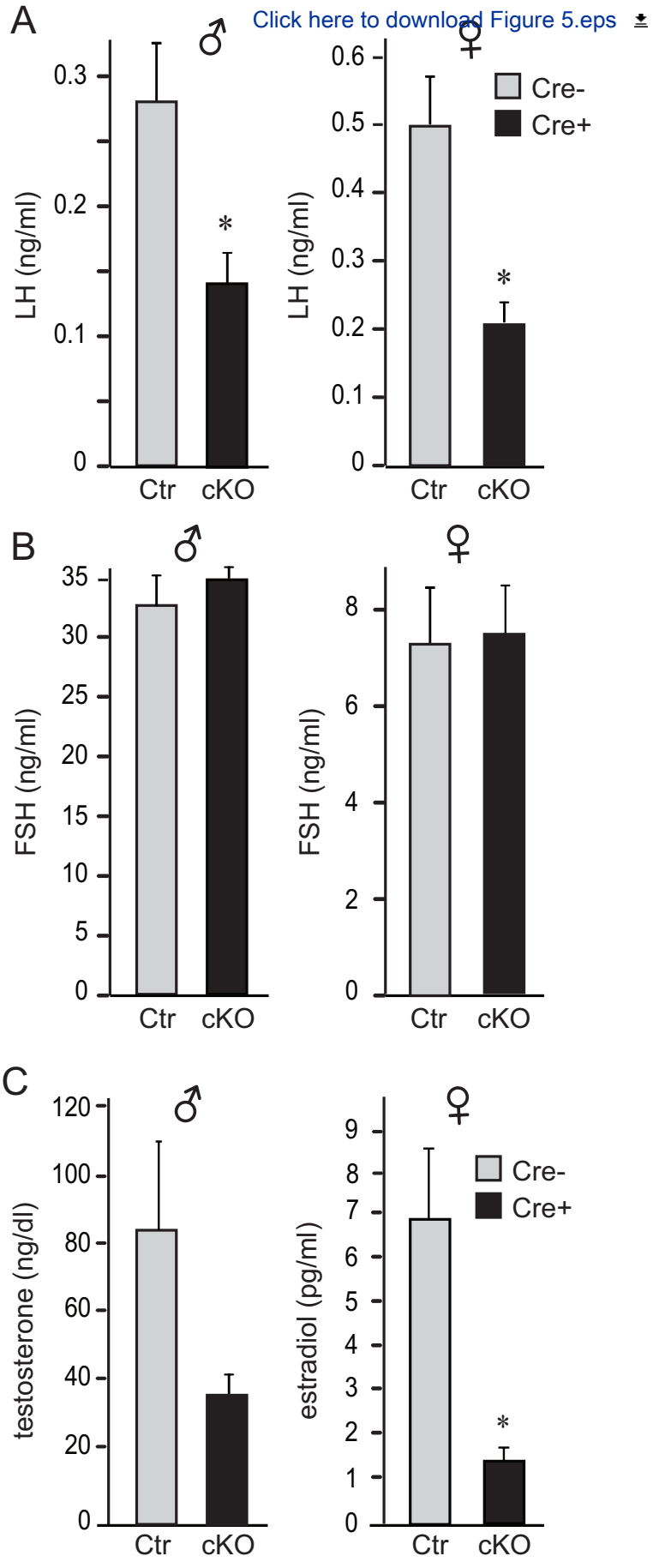
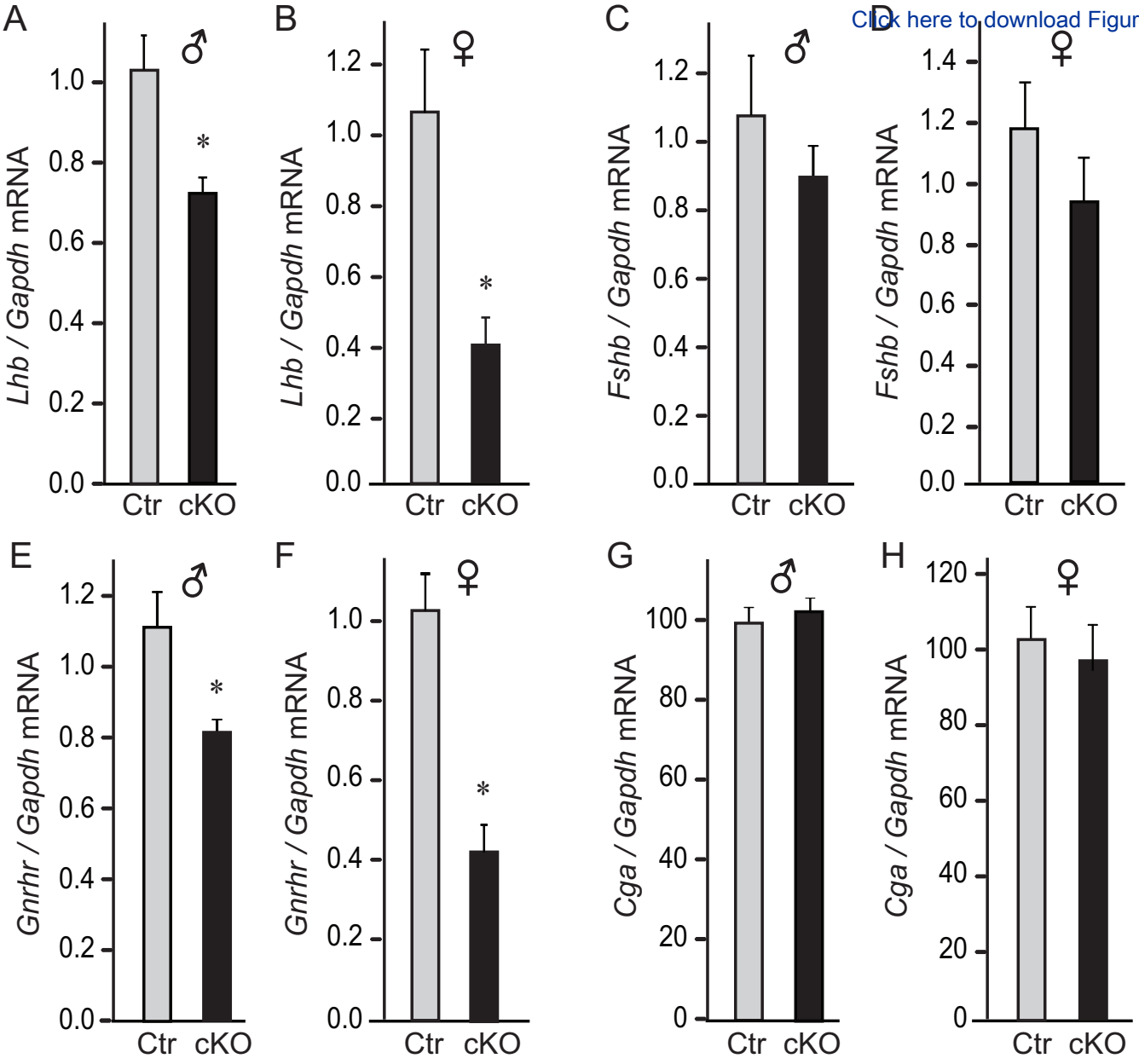


Figure 5

Figure 6



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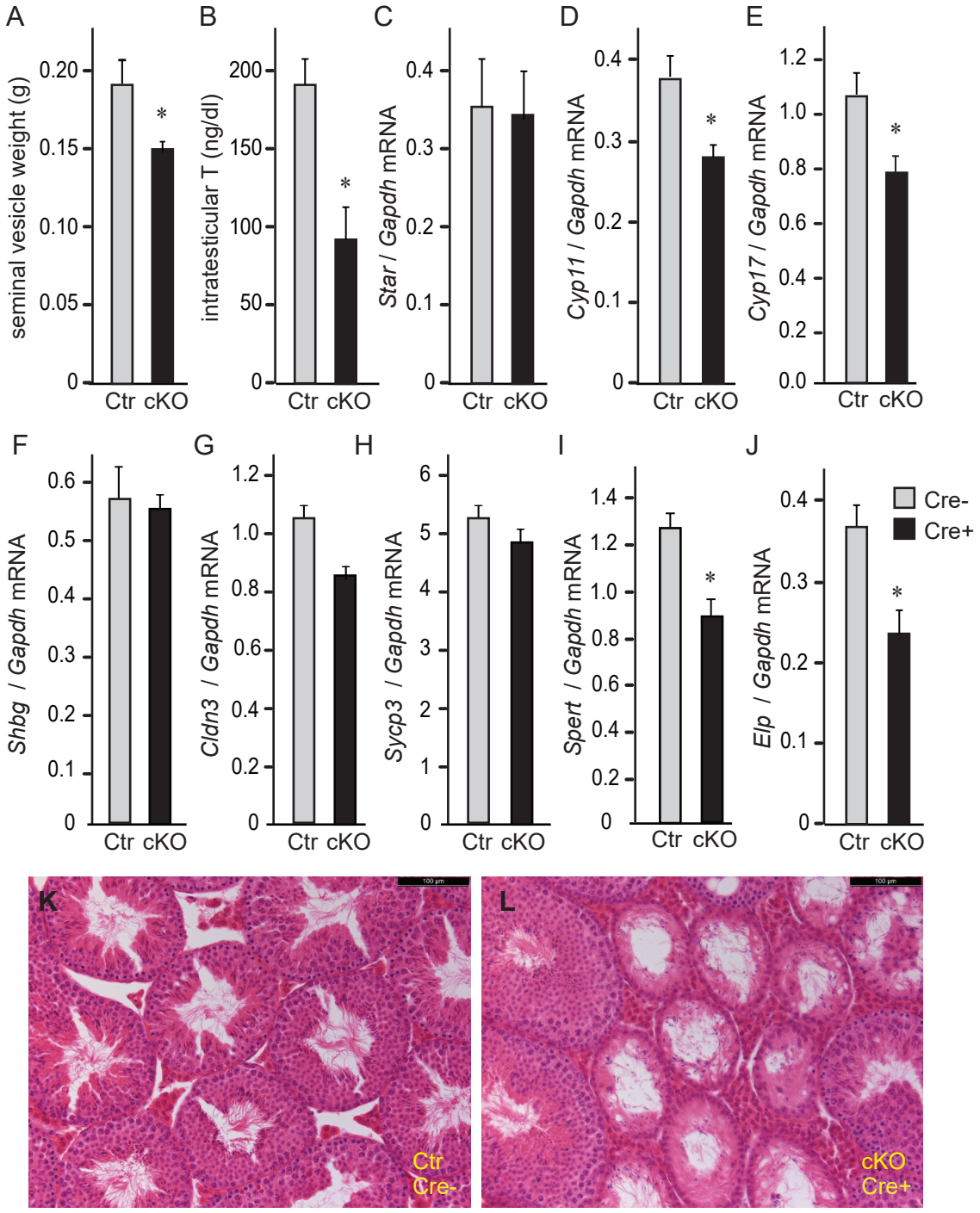

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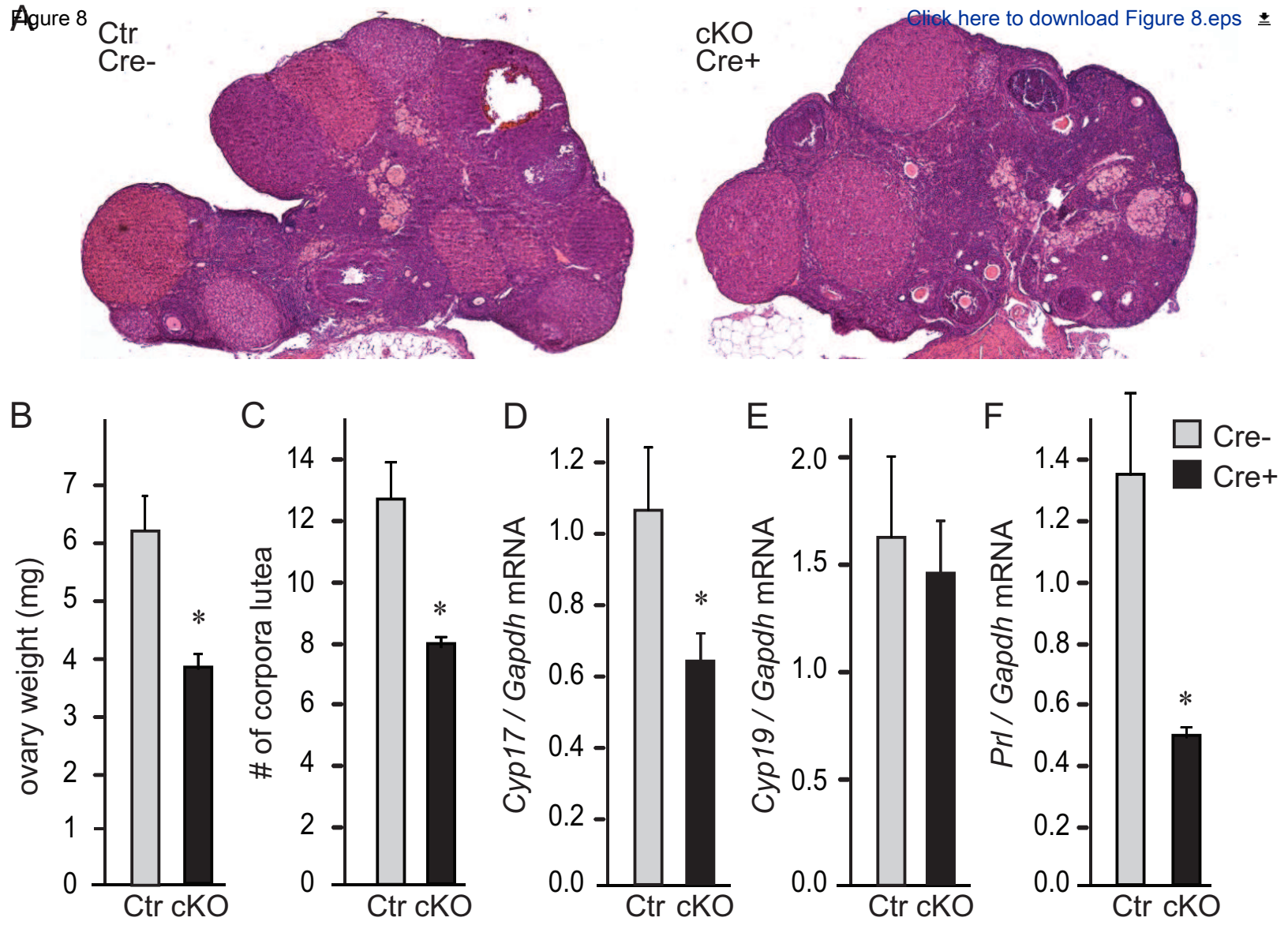

Figure 8

Figure 9

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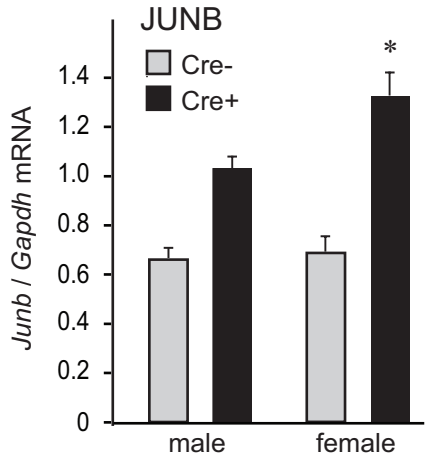

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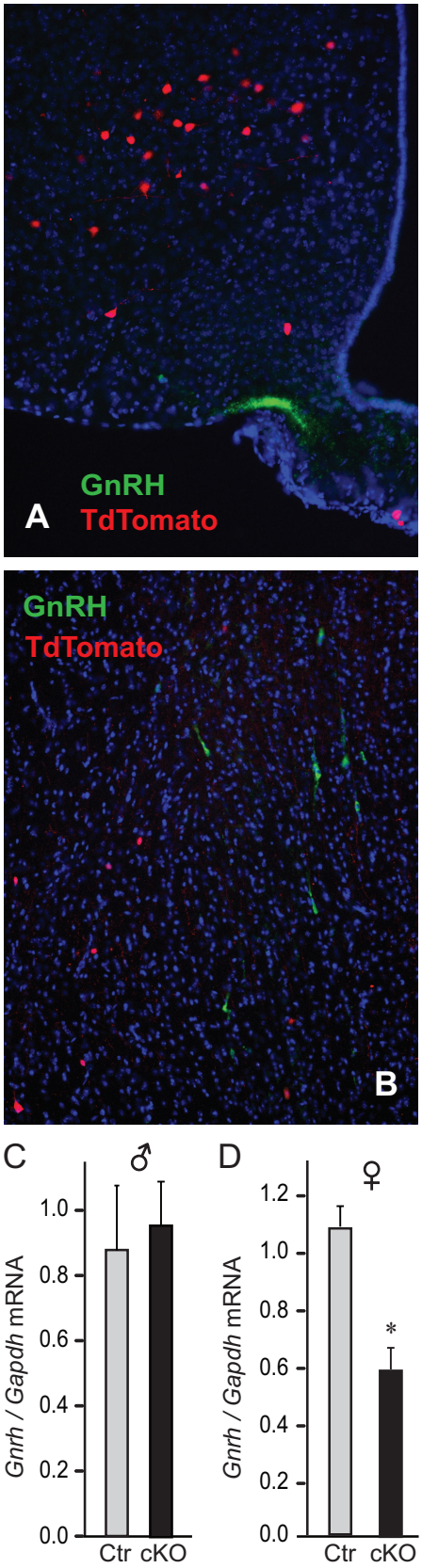

Figure 10